DOI: 10.7589/2016-11-244

*Journal of Wildlife Diseases*, 53(3), 2017, pp. 625–629


# Differential Susceptibility of Bighorn Sheep (*Ovis canadensis*) and Domestic Sheep (*Ovis aries*) Neutrophils to *Mannheimia haemolytica* Leukotoxin is not due to Differential Expression of Cell Surface CD18

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ABSTRACT: Bighorn sheep (*Ovis canadensis*) are more susceptible to pneumonia caused by *Mannheimia haemolytica* than are domestic sheep (*Ovis aries*). Leukotoxin produced by *M. haemolytica* is the principal virulence factor involved in pneumonia pathogenesis. Although leukotoxin is cytolytic to all subsets of ruminant leukocytes, neutrophils are the most susceptible subset. Bighorn sheep neutrophils are four- to eightfold more susceptible to leukotoxin-induced cytolysis than are domestic sheep neutrophils. We hypothesized that the higher susceptibility of bighorn sheep neutrophils, in comparison to domestic sheep neutrophils, is due to higher expression of CD18, the receptor for leukotoxin on leukocytes. Our objective was to quantify CD18 expression on neutrophils of bighorn sheep and domestic sheep. Cell-surface CD18 expression on bighorn sheep and domestic sheep neutrophils was measured as antibody binding capacity of cells by flow cytometric analysis with two fluorochrome-conjugated anti-CD18 monoclonal antibodies (BAQ30A and HUH82A) and microspheres. Contrary to our expectations, CD18 expression was higher ($P<0.0001$) with monoclonal antibody BAQ30A and was higher ($P<0.0002$) as well with monoclonal antibody HUH80A on domestic sheep neutrophils in comparison to bighorn sheep neutrophils. These findings suggest that the higher in vitro susceptibility to leukotoxin of bighorn sheep neutrophils compared to domestic sheep neutrophils is not due to higher expression of the leukotoxin receptor CD18 on bighorn sheep neutrophils.

*Key words:* Bighorn sheep, CD18, domestic sheep, flow cytometry, leukotoxin, *Mannheimia haemolytica*, *Ovis canadensis*, pneumonia.

Pneumonia is the primary disease responsible for the drastic decline of bighorn sheep (*Ovis canadensis*) populations in North America (Miller 2001). Bacteria commonly isolated from pneumonic bighorn sheep lungs include *Mannheimia haemolytica*, *Bibersteinia trehalosi*, *Pasteurella multocida*, and *Mycoplasma ovipneumoniae* (Besser et al. 2008; Wood et al. 2017). Although *M. haemolytica* causes pneumonia in other ruminants such as cattle, goats, and domestic sheep (*Ovis aries*), bighorn sheep appeared to be far-more susceptible hosts (Foreyt 1994; Dassanayake et al. 2009). *Mannheimia haemolytica* produces several virulence factors; however, studies with leukotoxin-deleted mutants have confirmed leukotoxin as the most important virulence factor contributing to the development of pneumonia (Tatum et al. 1998; Dassanayake et al. 2009).

Previous studies identified $\beta_2$-integrins as the receptor for leukotoxin on ruminant leukocytes (Ambagala et al. 1999; Dassanayake et al. 2007). Leukotoxin-induced cytolysis of transfectants exhibiting monomeric expression of the β subunit CD18, but not the α subunit CD11, confirmed CD18 as the functional receptor for *M. haemolytica* leukotoxin (Dassanayake et al. 2007).

There is a strong correlation between the degree of leukotoxin-induced cytolysis and CD18 expression on target cells (Deshpande et al. 2002). Increased CD18 (LFA-1) expression on bovine polymorphonuclear neutrophils (PMNs) following incubation with cytokines also results in increased leukotoxin binding and cytolysis (Leite et al. 2002). Leukotoxin induces cytolysis of all subsets of leukocytes, but PMNs are the most-sensitive subset. Furthermore, bighorn sheep neutrophils are four- to eightfold more susceptible to leukotoxin-induced cytolysis than are domestic sheep neutrophils (Silflow and Foreyt 1994). These observations prompted us to

hypothesize that the higher susceptibility of bighorn sheep neutrophils is due to the higher cell surface expression of the leukotoxin receptor CD18. To test this hypothesis, CD18 expression on neutrophils from bighorn sheep and domestic sheep was quantified by flow cytometry.

All experimental protocols were approved by the Washington State University Institutional Animal Care and Use Committee. Four captive bighorn sheep and four domestic sheep at our facility were selected as blood donors. All the animals were clinically healthy and approximately 2–3 yr old at the time of blood collection. Peripheral blood samples were collected via jugular venipuncture into ethylenediaminetetraacetic acid tubes. Polymorphonuclear neutrophils were isolated from blood by standard density gradient centrifugation and hypotonic lysis of red blood cells. Isolated PMNs were resuspended in RPMI-1640 medium (Invitrogen, Carlsbad, California, USA) and kept on ice until needed. The PMNs isolated by this method is defined as "purified neutrophils." The viability of isolated PMNs was determined by trypan blue dye exclusion and cells $\geq$95% viable were subsequently used for the assay. Mouse monoclonal antibodies (mAbs) specific for bovine CD18 (BAQ30A; immunoglobulin G1) and human CD18 (HUH82A; immunoglobulin G2a), which cross-react with ovine CD18, were obtained from Washington State University Monoclonal Antibody Center. Both mAbs were directly labeled with Alexa Fluor 488 (Invitrogen) and then used to label CD18 on PMNs. We used fluorescein isothiocyanate-conjugated goat anti-mouse Ig (Invitrogen) as the control antibody in flow cytometry.

The purified neutrophils from both bighorn sheep and domestic sheep were incubated with anti-CD18 mAbs. Briefly, $1\times10^6$ cells in 100 µL of flow cytometry staining (FACS) buffer (3% horse serum and 0.01% sodium azide in phosphate-buffered saline, pH 7.4) were placed in a V-bottom, 96-well plate, and anti-CD18 mAbs (BAQ30A or HUH82A at 15 µg/mL final concentration) were added into each well. The plate was incubated on ice for 30 min in the dark. Following three washes in FACS buffer (400 × G for 5 min), cells were suspended in 200 µL of 1% formalin in phosphate-buffered saline (fixative) and analyzed by a flow cytometer (FACSort, Becton-Dickinson, San Jose, California, USA) under a gate specific for neutrophils. For each sample, at least 10,000 events were acquired and median, geometric mean, and peak channel values for CD18 were obtained using FCS Express software (DeNovo Software, Glendale, California, USA).

Similar to purified neutrophils, CD18 expression on "unpurified neutrophils" was also assessed. Briefly, 100 µL whole blood from bighorn and domestic sheep were incubated with anti-CD18 mAbs (BAQ30A or HUH82A at 15 µg/mL, final concentration) for 30 min on ice, and red blood cells were removed by hypotonic lysis. Cell pellets were suspended in 200 µL of fixative and analyzed by a flow cytometer under a gate specific for neutrophils.

Although flow cytometry data enable us to identify CD18 fluorescent intensities, it is inadequate to calculate CD18 numbers on neutrophils. To quantify CD18 expression, Quantum Simply Cellular (QSC®, Bangs Laboratories Inc., Fishers, Indiana, USA) anti-mouse immunoglobulin G kit was used as described by the manufacturer. The regression curves obtained with microspheres were used to quantify CD18 mAbs bound to the neutrophils and expressed as antibody binding capacity values (ABC) of the cells. Three independent experiments on three separate days were performed to label CD18 on PMNs with both mAbs. Data were analyzed using the MIXED procedure from SAS (SAS Inc., Cary, North Carolina, USA). The model included the fixed effects of species (bighorn sheep or domestic sheep), cell source (purified PMNs or unpurified PMNs from whole blood), collection time points (times 1, 2, and 3 on three separate days), and the interaction between species and cell source. The animal was included in the model as a random effect. The term "significant" indicates a value of $P<0.05$.

The relative density of CD18 on neutrophils was measured as the antigen binding

TABLE 1. Comparison of CD18 expression levels on neutrophils. Purified neutrophils (by density gradient centrifugation) and unpurified neutrophils (in the whole blood) were incubated with Alexa Fluor 488 directly conjugated anti-CD18 mAbs (BAQ30A or HUH82A) and analyzed by a flow cytometer under a gate specific for neutrophils. The CD18 expression levels on neutrophils were calculated by regression curves obtained with Quantum™ Simply Cellular® microspheres and expressed as antibody binding capacity values of the cells. Results shown are the means and SE of antibody binding capacity values of the neutrophils for CD18 from three independent experiments. The CD18 expression levels on neutrophils were compared between species (bighorn sheep [*Ovis canadensis*] vs. domestic sheep [*Ovis aries*]) and also the purification status (purified vs. unpurified). *P*-values <0.05 were considered significant.

| | Mean±SE antibody binding capacity (relative fluorescence activity) | | | |
|---|---|---|---|---|
| | BAQ30A | | HUH82A | |
| Species | Purified neutrophils | Unpurified neutrophils | Purified neutrophils | Unpurified neutrophils |
| Bighorn sheep | 71,487±3,010[a] | 67,133±5,998 | 89,173±4,207[b,c] | 76,675±6,953[c] |
| Domestic sheep | 98,207±3,010[a,b] | 70,364±5,998[b] | 120,289±4,207[a,b] | 79,434±6,953[a] |

[a] Statistically different ($P<0.0001$).
[b] Statistically different ($P<0.0002$).
[c] Statistically different ($P<0.0018$).

capacity values of the cells (ABC) for two mAbs (BAQ30A and HUH82A), expressed as mean±SE. Fluorescence intensities exhibited by bighorn sheep and domestic sheep PMNs were different, indicating differences in CD18 expression levels on bighorn sheep and domestic sheep PMNs. Contrary to our hypothesis, cell surface CD18 expression on purified domestic sheep neutrophils labeled with BAQ30A mAb was higher ($P<0.0001$) than the similarly labeled bighorn sheep neutrophils (Table 1). Similar to BAQ30A mAb, higher ($P<0.0002$) CD18 expression in domestic sheep neutrophils was also observed with HUH82A mAb as compared to bighorn sheep (Table 1).

The CD11/CD18 dimer is mainly stored in secondary granules and translocated to plasma membrane upon activation. The receptors CD11b, CD18, and CD62L on PMNs are susceptible to artefactual changes during cell isolation process. To evaluate this possibility, unpurified neutrophils in the whole blood were directly labeled for CD18 and immediately assessed by flow cytometry. A reduced ($P<0.0002$) level of CD18 expression on unpurified neutrophils in comparison to purified neutrophils was detected for both anti-CD18 mAbs for domestic sheep (Table 1), but only HUH82A mAb showed such reduction for bighorn sheep (Table 1). In contrast, no significant difference in CD18 expression levels was observed when comparing unpurified neutrophils for both anti-CD18 mAbs between bighorn sheep and domestic sheep (Table 1).

The PMNs are the most-abundant peripheral white blood cell types and in bighorn sheep account for approximately 75% of peripheral blood leukocyte population (Highland et al. 2016). Neutrophils are the first cell type to respond to sites of infection-inflammation and their bactericidal activity is crucial to eliminate invading bacterial pathogens. Alveolar macrophages play a critical role when eliminating pathogens invading the lungs. Domestic sheep alveolar macrophages produce significantly higher levels of arachidonic acid metabolites such as thromboxanes, leukotrienes, and hydroxyeicosatetraenoic acids when compared to bighorn sheep alveolar macrophages following incubation with agonists (Silflow et al. 1991). However, bighorn sheep alveolar macrophages produce higher levels of prostaglandins metabolites. While leukotrienes such as $LTB_4$ are known to recruit neutrophils, prostaglandins such as prostaglandin $E_2$ ($PGE_2$) could suppress the bactericidal activity of alveolar macrophages. Our previous lamb inoculation study revealed that defective *M. haemolytica* clearance in bighorn sheep lungs was responsible for

enhanced lung pathology in comparison to domestic sheep (Subramaniam et al. 2011). Taken together, differences in $LTB_4$ and $PGE_2$ levels and bacterial clearances are the most-likely reasons for observed higher lung lesions in bighorn sheep in comparison to domestic sheep upon inoculation with *M. haemolytica* (Subramaniam et al. 2011).

In resting neutrophils, $\beta_2$-integrin CD18 (LFA-1) is inactive to interact with endothelial cell adhesion molecules (example: ICAM-1 and ICAM-2). The $\beta_2$-integrins get activated bidirectionally through "outside-in" and "inside-out" signaling. Inside-out signaling originated in the cytosol, which is typically mediated through ligation of G-protein-coupled receptors such as CXC chemokine receptors that bind to interleukin-8 (IL-8), leading to conformational changes of LFA-1 which in turn increases the affinity of LFA-1 to ICAMs. This activation process does not need increased LFA-1 expression on leukocytes but only the conformational shift in LFA-1. Using a panel of mAbs, Beals et al. (2001) demonstrated the presence of activation epitopes in the CD18 subunit in human T-cells. We previously showed that bighorn sheep PMNs expressed several-fold higher levels of IL-8 than those of domestic sheep upon stimulation with heat-killed *M. haemolytica* or lipopolysaccharides (Herndon et al. 2010). Therefore, it is tempting to speculate that bighorn sheep neutrophils are more prone to activation by inside-out signaling and subsequently to leukotoxin-induced cytolysis than are domestic sheep neutrophils. However, the lack of mAbs to identify activated CD18 in ruminants prevented us from evaluating such LFA-1 activation, conformational changes, and sensitivity to leukotoxin. Based on the findings from our study, we can only conclude that the higher sensitivity of bighorn sheep neutrophils for *M. haemolytica* leukotoxin, in comparison to domestic sheep neutrophils, is not due to the CD18 expression level differences.

This research study was supported by funds from Morris Animal Foundation, the Foundation for North American Wild Sheep, and its Chapters.

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*Submitted for publication 4 November 2016.*
*Accepted 10 January 2017.*